ROPINESS IN WATTLE BARK INFUSIONS.

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(With Plate ix.)

The development of ropiness in tan liquors is occasionally met with and probably would be of more common occurrence but for the fact that tanners have learnt by experience how to treat their liquors in order to avoid this objectionable fermentation. The phenomenon does not appear to have been examined, or at least no information about such ropiness has been published. Doubtless the reason for this lies in the difficulty of isolating an organism, capable of producing ropiness, from such a population of diverse organisms as must be present in a fluid with the history of tan liquor. But, beyond this fact, there is something about the subject that is peculiar, as was shown before this investigation was begun. A tan liquor claimed to be ropy was sent to the laboratory and upon being examined no ropiness could be detected. It was quite limpid and, upon being tested for viscosity, showed a water ratio of 1.0714 at 19°. When allowed to flow through a fine capillary, 100 c.c. of the reputed ropy liquor ran through in 210 seconds as against 196 seconds for distilled water. The liquid was plated and the bacteria examined, but no ropy organisms could be detected.

The Isolation of the Bacteria.

On account of the diversity of the tan liquor flora and the difficulty of obtaining a good specimen of ropy liquor at the time, the writer considered that the problem could best be attacked from the side of the wattle bark. There promised to be more chance of obtaining an organism capable of causing the ropy fermentation of raw bark infusions than of isolating a similar organism from a ropy tan liquor. As a matter of fact, during some experimental work, ropiness did develop in a bark infusion and a search showed, among many colonies, one possessing a ropy consistency upon a plate of dextrose medium. The organism also caused a fluid medium containing dextrose to become ropy. It was reserved until occasion permitted its further examination.

Some months later, it was decided to isolate fresh races of the ropy organism. Fragments of wattle bark (*Acacia pycnantha*) were put into bottles and covered with water and sometimes with nutrient liquids. Two bottles out of many showed ropiness. One of them had received raw tap water, the other boiled tap water with 0.25 % meat-extract. It was evident that the bacterium was to be found in the bark and was not derived from an outside source such as the tap water and, from the great number of bottles that were prepared, it was shown that the bacteria were not to be found on every bit of bark. A further test as to the absence of the bacteria in tap water was made by filtering a quantity of water and using the slime that adhered to the candle in conjunction with sterilised and raw barks. No ropiness developed in either case.

It was noted, but this may be of no importance, that the ropy infusions were slow to show growths of moulds on the surface of the fluids, while all the non-ropy tests soon became covered with mould. Possibly the ropy bacterium utilised all the available carbohydrate.

The ropy fluids in both of the positive tests contained many bacteria capable of forming slime on dextrose media, but only one of the numerous forms produced ropy colonies and the same organism was found in both positive tests. It gave a canary-coloured, loose, slightly raised growth on nutrient agar, and under the microscope appeared as a round yellow colony with central granules and homogeneous periphery. The bacterium was grown in bouillon and after some time another bacterium of identical form but with different cultural characters appeared. This was at first supposed to be an impurity in the original colony but the same supposed impurity appeared in both races and in others obtained at a later date from bark. The supposed impurity was subsequently recognised as a phase of the original organism, A1.

The ropy organism first isolated and set aside for future examination will be referred to as B., the most active phase of which is B2. The organism isolated later and obtained several times from wattle-bark will be called A. Both bacteria exist in phases best recognised by the appearance of the colonies growing on the surface of nutrient agar. There is the primary phase, such as A1, a weak rope-producer, which can be altered into the strong rope-producer, A2. The change of phase is not abrupt and transition phases are met with. Some of these, generally yellow in colour by transmitted light, are closer related to A1, others, grey or smoky, are nearer akin to A2. The phase B1 was occasionally noted but, as compared with A2, B2 is remarkably stable.

The ropy bacterium, mixed possibly with the altered phase, readily developed ropiness in nutritive solutions containing dextrose, but did not have any apparent action upon a sterilised infusion of wattle bark. In case the infusion had been altered by the sterilisation and become unsuitable for the development of the ropy substance, the bacteria were grown in pasteurised infusion, then in infusion sterilised by filtration through porcelain and finally in raw infusion, but in none of these was there any trace of ropiness. This was rather aggravating but quite in keeping with certain earlier attempts to transfer the ropiness of the original infusion to bottles containing healthy infusions. Unless a mass infection were made, the ropiness could not be transferred, and one had the suspicion that the ropy substance had not increased, but had simply become more diffuse.

The explanation of the apparent anomaly was found after it had been shown that the slime or ropy material was coagulated by tannic acid. It follows from this observation that in the original case the ropiness had developed before much tannin had passed into the water, otherwise the slime produced by the bacteria would have been coagulated upon the bodies of the bacteria, and would have prevented them becoming distributed in the bulk of the liquid. To prove the reasoning, wattle bark, sterilised at 130°, was covered with water, seeded with the bacterium, B2, and incubated at 28°. In sixteen hours a ropy infusion was obtained. A repetition gave the same result.

So far we have arrived at the stage that ropiness is developed in weak infusions of bark substance and not in strong, and it remained to determine the amount of tannin which would permit or prohibit the production of slime.

On account of the inability to obtain a pure tannin, tannic acid was employed in the experimental work with synthetic media. Wattle bark infusion contains

tannin with certain nutritive substances. These consist partly of salts, partly of nitrogenous bodies, probably amido-acids akin to asparagin and partly of sugar. The latter is either free or so loosely combined with the glucosidal tannin as to be readily fermentable by yeasts or by *B. coli communis*.

A saline solution containing 2 % dextrose, 0.2 % asparagin and mixed salts was treated with increasing quantities of tannic acid and portions were seeded with the phase B2. The portion with 5 % of tannic acid showed a growth of bacteria but there was no evidence of ropiness. The portions with 2 % and less were ropy in 16 hours. Twenty-five days afterwards, the portion with 1 % was ropy, the others were not and contained flocculent sediments. This experiment indicated that the organism could develop ropiness in fluids containing dextrose and up to 2 % of tannic acid. This amount seemed to be the limit, as in course of time it slowly coagulated the ropy substance.

Experiments with Infusions of Bark.

An infusion of wattle bark was sterilised by filtration through porcelain. It had a Sp.G. of 1.026 at 22° which is roughly equivalent to 5 % of tannin. Portions of this infusion were diluted and seeded with bacterium A, subsequently found to be a mixture of A1 and A2. Ropiness appeared in 16 hours with the quarter strength while the half strength was unaltered. An extension of this experiment with more graduated strengths was made with the results as shown in the table.

Table i.— *Diluted Infusion of Bark.

Days at 28°	1	2	3	6
Bark infusion, diluted. Sp. G., 1.026.				
10 to 100	S	S	O	O
15 to 100	—	—	S	O
20 to 100	+	—	+	O
25 to 100	O	S	+	O
30 to 100	O	S	—	O
35 to 100	O	S	+	O

*In this and subsequent tables, "S" indicates a slight ropiness, the fluid giving threads varying from one-sixteenth to one-eighth of an inch in length. "+" indicates threads of one quarter of an inch or longer. "O" means no apparent ropiness and, in some cases, no growth.

The small amount of ropiness obtained with water containing 10 % of the infusion may have been due to the paucity of nutrients and the slower appearance of the ropiness with 25 % and over was possibly caused by the retarding action of the tannin which appeared to have coagulated the ropy material by the 6th day. The disappearance of the ropiness may not have been entirely due to the coagulation of the ropy substance, for other experiments with acids and with salts, which will be described later, gave indications of a digestion or solution taking place.

Some time afterwards, eighty days to be exact, the filtered extract which had thrown a deposit was diluted with water in the proportion of three parts of

extract to seventeen of water, thus bringing the original approximately 5 % of tannin down to about ¾ %. The solution was divided into three sets, each set containing a control and two other portions, one with 0.2 % and one with 0.4 % of calcium lactate. The sets were seeded with phases A1, A2 and B2. Phase A1 did not become ropy. Phase B2 developed ropiness in the control only, while phase A2 showed ropiness in all tests. In these, the ropiness did not appear until the third day at 28° and it had disappeared by the sixth, giving place to cob-webby growths consisting of bacteria enmeshed in coagulated slime. The experiment showed that ropiness may develop in dilutions of old extract of wattle-bark and that it soon disappears. It also seemed to show that calcium lactate, a substance probably occurring in old tan liquors, has little or no influence in assisting the ropy fermentation.

During the investigation the bacteria were tested to see if they retained the power of making infusions of wattle bark ropy. Their physiological activities were being tested in synthetic solutions, and in these the characteristic ropiness was being produced, but it was considered advisable to prove that this also happened in bark infusions. Thirty gram portions of raw bark were put into sterile 4-ounce bottles and 50 c.c. portions of sterile water were added. The water just covered the bark. The liquids were seeded with the bacteria and incubated at 28°. Upon the first occasion of this routine testing, phase A1 produced the characteristic ropiness in a day and phases B2 and A2 in two days.

Twelve days later the test was repeated. Phases B2 and A2a developed the ropiness in two days, a duplicate race of B2 in three days. By the fourth day, phase A2 had developed ropiness. Phase A1, which had given a positive result twelve days before, was negative.

Other tests made from time to time showed, like the above, a certain variability in the activity of the phases. This was to be expected, for a stock culture could not be kept on account of the alteration of one phase into another. The bacteria were carried over from colony to colony, that is, plates were smeared every few days and from these, colonies were picked out and seeded into bouillon. It was only by proceeding in this way that the phases A1 and A2 could be maintained in a pure state.

When infected bark is covered with water and allowed to stand, the bacteria grow and produce the ropy substance, while the solution increases in strength. The bacteria apparently grow in clumps of slime, that is to say, they form a coherent slime and remain imbedded in this slimey environment. This is demonstrated when the bacteria are grown in saccharine nutrient solutions containing chalk; the blobs of cohesive slime can be seen upon rotating the flask, and they are incapable of being broken up by the rotation of the flask. Once the blob of slime around the bacteria is admitted, it becomes a matter of question as to the diffusive speed of the tannins and non-tannins through the slimes, just as it is a question about the diffusive speed of the non-tannins and tannins from the bark.

If in making an extract, the non-tannins, which we will presume are chiefly bacterial nutrients are the first to diffuse, or preponderate in the initial diffusion, the bacteria will grow and, in doing so, form a protective slime envelope which may be protective until the tannins become sufficiently concentrated to coagulate it. Such a coagulation occurs experimentally in dilute infusions of bark, but it has not been observed to occur in cases where the bark has been covered with water, and the infusion allowed to remain in contact with the bark.

In an endeavour to throw some light upon this matter, portions of raw bark were treated with water in the ratio of three of bark to five of water, and after

contact for varying times, the infusions were filtered. The following were the Specific Gravities of the extracts:—15 minutes, 1.010; 30 minutes, 1.013; 1 hour, 1.016; 2 hours, 1.020; 3 hours, 1.024; 4 hours, 1.027; 5 hours, 1.029; 1 day, 1.048; 3 days, 1.052. The infusions were portioned into tubes and seeded with phases of the bacteria.

Phase A1: no ropiness in any of the extracts.
A2: ropiness in all up to three hours.
B2: ropiness in all up to 24 hours.

The experiment was repeated with new extracts up to five hours.

Phase A1: no ropiness in any of the extracts.
A2: ropiness in all extracts.
B2: ropiness in all extracts.

From the earlier tests with bark extract, it appeared probable that tannin when present in excess will prohibit the formation of the ropy substance. But we are in doubt as to just how much will constitute an excess. Tannic acid seemed to act differently from the tannin in bark extract, and it is possible that ropiness occurs when there is a balance between the tannins and non-tannins of the extracts. Several experiments were made with the idea of feeling the way in this direction.

A quantity of bark was infused for two days at 28° with twice the weight of water. The infusion had a Sp. G. of 1.053. Portions were seeded with the various phases of the bacteria and in no case was ropiness obtained. The extract was probably too rich in tannin to permit the formation of the slime. It was then progressively diluted down to one-tenth the strength and seeded with phases A2 and B2. No ropiness became apparent. Bearing in mind the earlier experiment with the timed infusions of bark, in which the five hours' infusion having a Sp. G. of 1.029 became ropy, it seems that this longer infusion, after dilution to an approximate Sp. G. of 1,005, failed to produce ropiness because the tannins overwhelmed the activity of the nutrients.

In another experiment bark was treated for two hours with twice its weight of water and filtered. A quantity of water equal to that removed was added to the residual bark and allowed to remain in contact for 22 hours. The two infusions were called "A" and "B" respectively. Infusion "A" contained 5 % of solids and 0.232 % of ash; infusion "B," 7 % of solids and 0.245 % of ash. The two infusions were mixed in descending and ascending proportions from 5 to 0 and from 0 to 5 and seeded with A2 and B2. The tests with B2 did not become ropy.

Table ii.—Medium and Strong Infusion of Bark.

Phase		A 2		
Days at 28°		1	2	3
"A"	"B"			
5	0	O	+	+
4	1	O	–	S
3	2	O	S	S
2	3	O	S	O
1	4	O	O	O
0	5	O	O	O

The experiment seems to indicate that as the water lies in contact with the bark, the nutrients and tannins diffuse out and the proportion of these is such that ropiness can develop. But after a time, the tannins begin to preponderate and the development of ropiness is prevented.

Infusion "A" was treated with increasing amounts of tannic acid and seeded with A2. Ropiness developed in the control, but not in the portions containing 0.5 % and over.

The Change in Reaction.

During the growth in synthetic media, the bacteria produce a small but definite amount of acid from the sugar. In the presence of glycerine the medium may remain unaltered or it may become less acid. For example, a ropy dextrose fluid showed +3.4° while the control was +1.7°. A bulk culture with glycerine had at the start +2° to methyl red, +5.5° to litmus and +8.5 to phenolphthalein. On the 6th and 12th days, when quite ropy, the same respective acidities were determined. This however is unusual, for in other cases the glycerine medium became alkaline, as will be seen in some experiments given in the pages that follow.

The effect of varying the original acidity upon the development of ropiness was tested in a few experiments.

A fluid containing glycerine, meat extract and salts was divided into 50 c.c. portions, and these were given progressive quantities of phosphoric acid before being seeded with the bacterium A which was probably a mixture of the phases A1 and A2. Ropiness appeared on the fourth day at 28° in the portions containing originally an acidity of from −3° to methyl-red (=+2.5° to phenolphthalein) to +6° (=11.5°) but not in those containing +16° (=21.5°) and over. The conditions were unchanged on the sixth day.

A similar test was made with dextrose in place of glycerine. After sterilisation the fluid showed −9° to methyl-red and +6° to phenolphthalein. The portions were acidified progressively and seeded with the mixed phases, A1 and A2.

Table iii. Dextrose with increasing acidity.

Phase	A1 + A2				
Days at 28°	1	2	3	4	7
Acidity to methyl-red					
−1° and under	O	+	+	S	O
+1°	O	+	+	+	O
+5°	O	+	+	+	S
+8°	O	+	+	+	+
+12°	+	+	+	+	O
+15° and over	flocules				

Portions in which the phosphoric acid was replaced by sulphuric and hydrochloric acids gave similar results.

The experiment shows that there is a certain range of original acidity from about +5° to +12° as shown by methyl-red which conditions a rapid production

of ropiness. When the acidity is greater the ropy material assumes the flocculent condition. In most cases the ropiness was evanescent and soon disappeared, a circumstance which was subsequently traced to the presence of the phase A1. The acidity in the presence of dextrose probably increased, for the portions with +15° of original acidity showed +20° at the end of the experiment.

Another test was made with glycerine, using 0.5 % of the hydrated phosphate of soda instead of the usual mixture of salts. The medium as prepared was neutral to methyl-red and portions were acidified with phosphoric acid.

Table iv.—Glycerine with increasing acidity.

Phase	A1			A2			Acidity to methyl-red
Days at 28°	2	4	11	2	4	11	17
Acidity to methyl-red							
+5°	O	–	–	–	+	–	+3°
+7.5°	S	+	+	+	+	–	–
+10°	+	+	+	+	+	–	+5°
+12.5°	+	+	+	+	+	–	–
+15°	O	S	+	+	+	+	–
+20°	+	+	+	O	S	+	+7°

On the eighth day the portions infected with phase A1 showed, in the case of the 12.5° and of the 15°, the presence of transition forms. The original acidity decreased as time went on; on the seventeenth day, the test with an original acidity of +5° had become +3°, +10° had become +5°, and +20° had become +7° to methyl-red.

The original acidity of a glycerine medium does not seem to have much influence upon the production of ropiness, but this may be explained by the fact that the acidity is reduced during the growth of the organism.

The acidity of spent tan liquors seems to vary from +12.5° to +20° by Procter's lime water test, and one which was tested showed +10.4° by this test and +5° by methyl-red. So far as mere acidity is concerned, the organism should produce ropiness in such an end-liquor, but when tested it did not do so.

The Disappearance of Ropiness.

The disappearance of the ropiness in culture fluids was noted first in the case of B2 when growing in a medium containing saccharose 2 %, meat extract 0.5 % and mixed salts (KH_2PO_4, 0.2 %; $MgSO_4$ Aq. 0.1 %; $CaCl_2$, 0.02 %) made neutral to methyl-red. The fluid was ropy on the fourth day at 28° and quite limpid on the 6th when the acidity had risen to +8°. Again the experiment with varying amounts of acid noted on p. 57 showed a solution or digestion of the ropy material in the case of A, a mixture of A1 and A2.

The speed in the digestion of the ropy substance was tested upon several occasions by growing the phases of the bacteria in medium containing 2 % of dextrose with meat extract and mixed salts at 28°. The bacterial phases had been picked from agar plates and were typical, that is to say, they were the

phases known as A1, A2 and A2a. From the plates they were seeded into nutrient broth and transfers were made daily. In this medium they doubtless altered in the one direction or the other, but the change was very much slower than when a sugar or glycerine was present. The first test with the dextrose medium was made one day after isolation from the plate, and during the growth the medium became ropy and then, after an interval, the ropiness disappeared and the liquid became limpid.

A1 became limpid on the 3rd day.
A2 15th day.
A2a 26th day.

The second test was made six days after the isolation of the phase.

A1 became limpid on the 3rd day.
A2 20th day.
A2a was still ropy on the 26th day.

The third test was made thirteen days after the isolation.

A1 became limpid on the 6th day.
A2 was limpid on the 22nd day.
A2a was still ropy on the 22nd day.
A1 + A2 was limpid on the 8th day.
A1 + A2a was limpid on the 8th day.

In this test the purity of the phases was examined in a few cases. On the 12th day, A1 contained a few pure typical colonies, and on the 19th day no bacteria were found in a large loop of the culture. The digestion of the slime is apparently a prelude to the disintegration or death of the bacteria. On the 12th day both A2 and A2a contained bacteria which grew as colonies with the tint of A1 but much more granular; the granular lumps radiated to the margin and became larger as they approached the edge. This was the transition stage between phase A1 and A2. On the same day, large loops taken from the mixed growths of A1 with A2 and A2a were found to be sterile. Phase A2 consisted of A1 with a few of A2.

Other instances of the solution of the ropy material will be seen in the experiments dealing with the saline and carbonaceous nutrients.

The phase A2a gives a more ropy colony on nutrient agar than A2 which is somewhat gelatinous and is not so elastic when touched with the needle. It is, however, difficult to discriminate between the two as, when free to grow, the phase A2 often preponderates. That is to say, a plate when smeared with a reputed culture of either A2 or A2a may show a preponderance of A2a in the comparatively thickly sown parts and A2 in the areas with few colonies. At times, the two phases seemed to be remarkably consistent in remaining true to phase. In the majority of the experiments, A2a, has been classified under the phase A2 for the sake of simplicity but where both A2 and A2a have been simultaneously tested, the original designations have been retained to indicate a duplicate test.

The earlier observations led to the belief that the disappearance might result from the formation of acid from the sugar but this was negatived by an experiment made with the idea of determining the nature of the acids formed in the presence of sugar. The medium contained dextrose, meat-extract, potassium chloride and chalk; it was seeded with a mixture of A1 and A2 then known as Bact. A. The liquid never became acid and the particles of chalk were freely suspended when the flask was rotated. It was first incubated at 37°, at which

temperature no ropiness developed. Then the flask was transferred to an incubator at 28° and the culture became strongly ropy, the brownish ropy blobs, one of which was about two inches in diameter, were clearly shown against the milky chalk suspension. The flask was returned to the incubator at 37° when the ropiness disappeared. Once more the ropiness appeared at 28°. These observations clearly show that a digestion of the ropy substance occurs at 37° in a neutral solution. An acid reaction of the medium is not essential for the digestion of the slime but it is possible that it may assist.

That the temperature has much to do with the speed of the digestion of the slime was shown in a test in which phase A1 was grown at 22° and at 28°. Both were ropy on the second day, the 28° test was limpid on the 5th and the 22° test on the 8th day.

The experiment with chalk suggested the secretion of a slime-dissolving enzyme by the phase A1. Probably this is so but when experiments were made in which old fluid cultures of A1 were added to lumps of the purified gelatinous slime of A2 in presence of an antiseptic, no solution of the slime was obtained.

It became evident that phase A1 was capable of forming and eventually digesting the ropy substance. It also rapidly dies out and it may be noted in this connection that it produces a more rapid liquefaction of gelatine. If the A2 phase is used originally, the ropy substance may persist, while if a mixture of the phases is initially present, digestion occurs, but at a later period than in the case of the pure A1 phase. It seemed to be entirely a question of the relative numbers of the two phases during the period of bacterial growth. Instances of the autodigestion of the ropy material will be found in the experiments with the various sugars and salts.

Change of Phase.

These observations led to testing the reversion or alteration of the phases. It had been noted that glycerine favoured the production of ropiness from phase A1 and that dextrose did not or, if it did, the ropy fluid subsequently became limpid. This was confirmed in experiments subsequently recorded with sugars, etc., where ropiness slowly developed and persisted in the presence of glycerine but did not persist when other sources of carbon were used.

A specific test was made with cultures of the phases A1 and A2 taken from pure colonies and grown in broth for one day before being seeded into the test bottles. Phase A1 was sown in a fluid containing glycerine, meat-extract and sodium phosphate while phase A2 was grown in dextrose with mixed salts as on p. 58. Both tests were ropy on the third day, and on the thirteenth day, phase A1 in the glycerine was quite ropy, while phase A2 in the dextrose was limpid. Plates were prepared on the thirteenth day and these showed that phase A1 consisted of a mixture of typical colonies of phases A1 and A2, and that phase A2 had been altered into more or less vacuolated colonies of phase A1.

The experiment conclusively showed that the phases were reversible.

The Action of Tannic Acid.

The bacterium B2 was peculiar in giving pronounced ropy solutions when seeded into infusions of wattle bark of increasing strength and little ropiness in synthetic liquids. The reason for this could only be explained by testing the various nutrients in the presence of the nearest approach to the tannins available, namely tannic acid. It may be that the tannins in wattle bark infusions

behave differently to commercial tannic acid and this should be kept in mind when interpreting the results obtained in testing the commercial acid.

A solution of dextrose, meat extract and mixed salts was prepared, and to portions quantities of tannic acid rising from zero up to 0.6 % were added before the addition of B2. That with 0.1 % gave a faint ropiness and those with 0.2 % to 0.5 % contained slimey strings. There was no pronounced ropiness in any of them.

As meat extract forms a precipitate with tannic acid, it was replaced by asparagin. In this solution, B2 produced ropiness in the presence of 0.1 % and 0.2 % of tannic acid. The control test and those with quantities greater than 0.2 % gave a good growth of bacteria but no slime.

The experiment was repeated with a slightly greater percentage of asparagin (0.2 %) and dextrose (3 %) with mixed salts. Phase A2 gave ropy liquids with the control and 0.1 % of tannic acid, but not with larger quantities. Phase B2 only produced feebly gelatinous surface rings with quantities of tannic acid up to 0.2 %.

A medium containing levulose, 3 %, asparagin, 0.2 % and potassium citrate, 0.1 %, was prepared and seeded with phases A2 and B2. The former was a very active slime producer when used and produced ropiness in the presence of quantities of tannic acid up to 0.5 % and a slight ropiness with 1 %. Phase B2 gave an evanescent ropiness in the flask containing 0.5 per cent only, and not in any of the others.

The influence of the original acidity of the medium was tested by means of a solution containing dextrose, asparagin and mixed salts. One set had an acidity to phenolphthalein of +17°, another was neutralised until the acidity was +2.5°. Both were seeded with phase B2. That with +17° gave no ropiness in the control, a slight ropiness with 1 % of tannic acid and a distinct ropiness with 0.2 %; larger amounts were negative. With +2.5°, ropiness developed in the control test only. Thus the production of ropiness was irregular. Phase B2 gave ropiness in the control with +2.5° and not with +17°; with +17° and a small quantity of tannic acid it produced a ropy fluid.

In these experiments with tannic acid, either dextrose or levulose had been used and with them a certain irregularity of effect had been obtained. It was therefore deemed advisable to test the effect of other sources of carbon. As will be seen later, the experiment with nitrogenous nutrients seemed to indicate that a maximum amount of ropy substance would be formed in the presence of asparagin or ammonium sulphate. Similarly, the saline experiments indicated that sodium succinate was a favourable salt. Accordingly, media were prepared containing asparagin or ammonium sulphate 0.25 %, sodium succinate 0.2 % and a source of carbon 2 %. Tannic acid to the extent of 0.5 % was added to each flask after infection, by which procedure a coagulation of the infecting droplet was avoided. When a drop of infected bouillon is added to a solution of tannic acid, the drop is coagulated and the contained bacteria are probably prevented from being dispersed freely in the liquid. It is possible that much of the irregularity in the previous experiments may have been due to this imprisonment of the bacteria.

The groups of flasks were seeded with A2 and B2. Another group was seeded with B1 but as a plate, smeared at the time of infection showed that the phase had become altered to B2, the group became a duplicate of B2. Phase B2 was pure, while A2 at the time of seeding contained 90 % of A2 and 10 % of A1.

Table v.—Sugars, etc. with 0·5% Tannic Acid.

Phase	A2								B2							
Source of Nitrogen	Asparagin				Ammonium Sulphate				Asparagin				Ammonium Sulphate			
Days at 28°	3	4	6	10	3	4	6	10	3	4	6	10	3	4	6	10
1. Dextrose	–	+	+	+	+	+	–	–	+	+	–	O	–	–	±	O
2. Levulose	±	±	+	+	±	–	–	–	(+ O	O	O	O	(O O	±	–	S O
3. Saccharose	–	±	+	–	±	–	–	–	–	S	S	S	(O S	O S	O S	O S
4. Maltose	O	O	O	O	–	–	–	–	O	O	O	O	O	O	O	O
5. Galactose	±	±	+	+	±	+	–	+	±	+	–	+	+	–	+	–
6. Raffinose	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
7. Mannit	–	–	+	–	–	–	+		–	–	–	S	O	O	O	S
8. Control	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

On the 4th day, the ammonium sulphate tests of A2 were plated to see how far the phases had altered.

Table va.—Percentage of Phase A2 in Ammonium Sulphate at 28°

	Start.	4 Days.	10 Days.
Mannit	90	90	15
Saccharose	90	65	15
Maltose	90	35	25
Raffinose	90	30	5
Galactose	90	20	0
Dextrose	90	10	0
Levulose) Control)	90	0	0

In all cases, except mannit, the cohesive phase A2 had become, by the fourth day, more or less altered to the diffuse phase A1, and it is rather extraordinary that those with a maximum proportion of the diffuse phase should have been ropy. It is possible that the ropy substance was formed before the alteration to the diffuse phase occurred and the gradual suppression of the phase A2 as shown by the relative numbers on the 10th day, bears out this idea. Another peculiarity is that while the maltose test with asparagin gave no ropiness, and with ammonium sulphate a pronounced ropiness on the 4th day, smears made on that day showed the same proportion of A2, viz. 35 %. From these proportions it would appear that ropiness has less to do with the phase of the organism than the previous tests had led one to believe, but as on the 10th day, the asparagin test contained 1 % of A2, and the ammonium sulphate test 25 %, there is still the suspicion that some relation exists.

The duplicate tests of phase B2 were fairly concordant; differences were obtained with levulose and saccharose in conjunction with ammonium sulphate. With asparagin, a slight difference occurred in the case of levulose.

Phase B2, in this experiment, was shown to possess considerable activity in producing ropiness in the presence of tannic acid as compared with the preliminary tests, but it is possible that the saline constituents had much to do with the differences obtained.

In the experiment the media contained 0.5 of tannic acid and, as ropiness was obtained with this percentage, it was deemed advisable to see to what extent the most active phase could tolerate this acid. A medium containing dextrose 2 %, asparagin or ammonium sulphate 0.25 % and sodium succinate 0.2 % was portioned out and seeded with phase A2 taken from a colony two days previously. Then the various quantities of tannic acid were added.

Table vi.—Dextrose with increasing Tannic Acid.

Phase	A2							
Source of Nitrogen.	Asparagin.				Ammonium Sulphate.			
Days at 28°	1	3	6	10	1	3	6	10
Tannic Acid per cent.								
0·2	+	+	+	+	+	+	+	+
0·4	+	+	+	+	+	+	+	+
0·6	+	+	+	+	S	+	+	+
0·8	+	+	+	+	S	+	+	+
1·0	S	S	+	S	S	+	S	O
1·5	S	S	S	O	O	O	O	O
2·0	O	O	O	O	O	O	O	O

On the 6th day certain of the cultures were smeared on agar. With 0.2 % and 0.6 % of tannic acid, in the presence of asparagin, the colonies were of a novel type. They appeared as round, raised, glistening, buff-coloured colonies with a glutinous consistency. Microscopically they had dark centres from which dark tufted fibres radiated through a yellow matrix to near the margin. In 10 % of the colonies this structure blended into that of phase A2, part of the colony showing the fibrous structure at one side and that of A2 at the other. They were clearly a transition phase of A2 more nearly related to A2 than to A1. With 1 % and 2 % of tannic acid the colonies consisted entirely of the phase A1.

By the 12th day, the medium containing asparagin with 0.2 % of acid showed 96 % of A1, 2 % of the fibrous transition form of A2, and 2 % of A2. With larger amounts of acid the cultures contained very few living bacteria but they were of the kinds noted on the 6th day.

The cultures containing ammonium sulphate were tested on the 12th and 16th days. They contained few bacteria; with 0.2 % of acid, they consisted of the introduced phase A2 and, with larger quantities, they were the A1 phase.

The tendency of the tannic acid is to alter the phase A2 to A1 but this probably occurs after the ropy substance has been formed in the medium.

The experiment showed that a fairly active culture of the phase A2 could produce ropiness in the presence of quantities of tannic acid up to 1 % with ammonium sulphate, and up to 1.5 % with asparagin.

The sugar test with 0.5 % of tannic acid showed that galactose was a useful sugar for inducing the formation of ropiness, and an experiment was made to see the effect of increasing quantities of tannic acid in the presence of this sugar. The medium contained galactose 2 %, asparagin 0.25 %, sodium succinate 0.1 %, and sodium phosphate, anhydrous. 0.2 %. The phases were plated at the start and found to be pure.

Table vii.—Galactose with increasing Tannic Acid.

Phase	A1					A2					B2				
Days at 28°	2	4	6	9	13	2	4	6	9	13	2	4	6	9	13
Tannic Acid per cent.															
0·25	O	O	O	O	O	+	+	+	+	+	O	S	S	O	O
0·5	O	O	S	S	O	+	+	+	+	+	O	S	O	O	O
0·75	O	O	S	S	S	+	+	+	+	+	S	S	S	S	O
1·0	S	S	S	S	S	+	+	+	+	+	+	+	S	S	S
1·25	S	S	S	S	S	+	+	+	+	+	S	S	S	S	S
1·5	O	O	O	O	O	+	+	+	+	+	S	S	S	S	O
1·75	O	O	O	O	O	+	+	+	+	+	O	O	S	S	O
2·0	O	O	O	O	O	O	S	S	S	O	O	S	S	S	O
2·25	O	O	O	O	O	O	O	O	O	O	O	O	O	S	O
2·5	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

The flasks with 0.5 % and 1 % of tannic acid were examined several times by the plate method on nutrient agar with the following results:—

Table vii*a*. – Change of Phase.

Phase	A1		A2		B2	
Tannic Acid	0·5%	1%	0·5%	1%	0.5%	1%
Start	A1	A1	A2	A2	B2	B2
Four days	A1	A1	A2	A2	B2	B2
Nine days	A1 with 5% A2	A1	A2, 40% ; A2, trans. 30% ; A1, 30%	A1	B2, trans.	B2, trans.
Fourteen days	A1	A1	A2, 30% ; A2, trans. 60% ; A1, 10%	A1	B2	B2

It is clear from the experiment that the phase A1 is capable of producing ropiness in the presence of from 0.5 % to 1.25 % of tannic acid in a medium containing galactose. With 0.25 % there was no ropiness formed, and as the acid increased, so did the viscosity. Tannic acid or, possibly, acidity seems therefore to be a *sine qua non* for the formation of ropiness by this phase of the bacterium A.

The phase A2 at the time of the experiment was a strong slime-former, and gave a pronounced ropy solution in the presence of amounts of tannic acid up to 1 %. The ropy substance had been formed in the early days of the experiment, and it did not alter, although the phase in the case of 0.5 % of tannic acid changed to a mixture of phases, and in the case of 1 % it changed to A1.

The phase B2 gave ropiness in amounts of tannic acid up to 2.25 %, but the viscosity of the solutions was never so pronounced as in the case of A2. The most viscous solution was obtained in the presence of 1 % of tannic acid.

The experiment shows that the three phases which were tested produced ropiness in synthetic media when the tannic acid varied in amount up to 1.25 % or 2.25 %, and that the tendency of the bacterial phase A2 is to change, in the presence of tannic acid, to A1, and for the bacterial phase B2 to remain constant.

The same galactose medium was used to determine the comparative amounts of tannic acid and of phosphoric acid necessary to prevent the formation of ropiness by phase A2.

Table viii.—Tannic and Phosphoric Acids compared.

Phase		A2						
	Days at 28°	1	3	7		1	3	7
	Tannic Acid per cent.				Phosphoric Acid in degrees.			
1	0·0	+	+	+	0	+	+	+
2	0·25	+	+	+	3	+	+	+
3	0·5	+	+	+	6	+	+	+
4	0·75	+	+	+	9	+	+	+
5	1·0	S	+	+	12	+	+	+
6	1·25	S	+	+	15	S	+	+
7	1.5	S	+	+	18	S	+	+
8	1·75	S	+	+	21	S	+	+
9	2·0	O	O	O	24	S	+	+
10	2·25	O	O	O	27	S	+	+
11	2·5	O	O	O	30	O	O	O

Certain of the cultures were examined at the end of the first day, and they were found to contain the introduced phase, A2, in pure culture. They were again examined on the seventh day and it was found that the phase had altered to A1.

Table viii*a*.—Percentage Composition of the Phases (Seventh day).

	Tannic Acid			Phosphoric Acid		
	A1	A2	A2 trans.	A1	A2	A2 trans.
1	35	25	40	80	10	10
3	0	90	10	70	20	10
5	80	5	15	85	15	0
9	Sterile			75	15	10

The indication that a moderate quantity of tannic acid tended to maintain the stability of the introduced phase A2, led to the tannic acid tests being again examined on the 10th day, when the following percentage counts of the kinds of colonies were noted.

Table viii*b*.—Percentage Composition of the Phases with Tannic Acid (Tenth day)

	A1	A2	A2 (transition)
1	70	30	0
2	0	95	5
3	0	50	50
4	25	65	10
5	100	0	0
6*	90	10	0

*Scanty growth.

It appears that from 0.25 % to 0.5 % of tannic acid, when added to a synthetic medium such as was used, maintains the stability of the A2 phase and that smaller or larger quantities bring about its conversion to the less cohesive phase A1. In contrast, phosphoric acid does not appear to have much influence in maintaining the stability, for on the 7th day there was a 70 to 85 % conversion, irrespective of the amount of acid added.

The limiting amount of tannic acid in this synthetic medium for the phase A2 at the time of making the experiment was 2 % and of phosphoric acid +30° (equivalent to 30 c.c. of normal acid per litre).

The two last experiments with tannic acid in synthetic media indicated that 2 % of the acid prohibited the formation of the ropy substance. It appeared to be advisable to extend the line of experimentation and obtain some information regarding the action of tannic acid when added to an infusion of wattle-bark capable of giving ropiness. With this object in view an infusion was prepared by mixing three parts of water with two parts of bark, and filtering the liquid at the end of two hours. Portions were seeded with A2 and B2, and were treated with progressively increasing quantities of tannic acid. The portions seeded with A2 did not develop ropiness and when examined on the 4th day they were found to contain from 85 % to 95 % of A1. The portions seeded with B2 developed a pro-

nounced ropiness with quantities of tannic acid up to 0.4 % and a slight ropiness with 0.7 %. On the 4th and 9th days these contained a pure culture of B2.

The experiment was repeated a fortnight later with a similarly prepared infusion. It had a Sp. G. 1.023 at 21° and an acidity of +24° by Procter's lime water test. A similar infusion made on the following day at 21° had a Sp. G. 1.022, +22.5° by Procter's test and +7° to methyl-red. Procter's test seems to indicate the acids other than tannic acid that are present, for tannic acid in pure solution is precipitated at once by the lime water.

Table ix.—Bark Infusion with Tannic Acid.

Phase	A1				A2				B2			
Days at 28°	2	3	5	9	2	3	5	9	2	3	5	9
Tannic Acid per cent.												
0·0	O	O	O	O	+	+	+	+	S	+	+	+
0.1	O	O	O	O	O	O	S	S	S	+	+	+
0·2	O	O	O	O	O	O	O	O	S	+	+	+
0·4	O	O	O	O	O	O	O	O	O	+	+	+
0·6	O	O	O	O	O	O	O	O	O	O	O	O

We see that an infusion of wattle-bark having a Sp. G. of 1.023 is immune to the phase A1 and that the derived phase A2 is able to develop ropiness, but the addition of a small quantity of tannic acid, 0.2 %, prevents the formation. A smaller quantity, 0.1 %, permits a slight ropiness to appear. Phase B2 is more tolerant of tannic acid, the limiting amount of which lies between 0.4 % and 0.6 %.

Larger amounts of tannic acid were used but these are omitted from the table. The portions containing over 0.4 % showed cobwebby growths, doubtless consisting of bacteria bound up with coagulated slime.

As a bark liquor of Sp. G. 1.010 may contain 1.8 % of tannin and 0.5 % to 0.7 % of extractives (non-tannins), the infusion of Sp. G. 1.023 presumably contained about 4 % of tannin. The experiments show that this reputed 4 % of tannin had much the same effect in prohibiting the formation of ropiness as 1.75 % of pure tannic acid.

A stronger infusion of wattle bark of Sp. G. 1.054 when seeded with A2 and B2 did not develop ropiness, even when the infusion was strengthened by quantities of dextrose and ammonium sulphate rising to 5 % and 0.5 % respectively. This seems to indicate that the tannins are the prohibiting agents, and when they are present in sufficient amount, bacterial nutrients have little influence in assisting the development of ropiness.

A few tests had been made in the earlier part of the research to see if the quantity of sugar in synthetic media had any effect in increasing the formation of ropiness, but it was not definitely shown that the amount of ropiness was proportional to the sugar in the medium, or that any advantage would be gained by increasing the quantity over the usual 2 %. It seemed possible, however, that sugar might to some extent modify the action of tannic acid, and that an increase

in the sugar content might enable the organism to withstand a higher amount of tannic acid. To test the matter portions of fluid containing asparagin 0.5 %, and sodium succinate 0.2 %, were given increasing amounts of dextrose and of tannic acid. The portions were seeded with a drop of a bouillon culture of A2 which at the time of inoculation contained A2, 85 %, and A1, 15 %, as shown by plate culture.

Table x.—Increasing Dextrose and Tannic Acid.

Dextrose %	2			4			6		
Days at 28°	3	7	11	3	7	11	3	7	11
Tannic Acid									
1·0	S	—	+	+	+	+	+	—	—
1·25	S	S	S	S	—	S	+	+	S
1·5	S	S	O	S	S	O	S	—	O
1·75	S	O	O	S	S	O	S	+	O
2·0	O	O	O	S	S	O	S	S	O
2·25	O	O	O	O	O	O	O	O	O

The portions with 1.5 % of tannic acid were examined on the 8th day. That with 2 % of dextrose contained A1 with 5 % of A2, with 4 % of dextrose had A1 with 20 % of A2, and 6 % of dextrose had A1 with 25 % of A2.

To judge by the pronouncedly ropy tests, an increase in the sugar does appear to mask, to some extent, the action of the acid, for on the 3rd and 7th days the "+" indications rise with the amount of acid, but so far as slight ropiness is concerned, there is little difference between the 4 % and the 6 %. The increased sugar also prevents, or rather hinders, the conversion of the phase A2 to A1, and thus masks the action of the acid by enabling more ropy substance to be formed by the cohesive phase.

Sources of Nitrogen.

An early attempt to determine the most favorable source of nitrogen was made with solutions of dextrose and mixed salts containing amounts of nitrogen approximately equivalent to 0.2 % of asparagin. On the sixth day a mixture of phases A1 and A2 had produced ropiness to a greater or less degree with asparagin, meat-extract, peptone, and ammonium sulphate, but potassium nitrate gave little growth and no ropiness. A second test using one-sixth the quantities of nitrogen and replacing the dextrose by glycerin showed that ropiness had developed by the fifth day in the presence of all the above sources of nitrogen, and also of potassium ferricyanide. Thus in the presence of glycerin and mixed salts any of these sources of nitrogen will serve.

At a later date, a more comprehensive experiment was made with phases A1, A2 and B2, using 2 % of dextrose or glycerin, 0.3 % of potassium citrate and 0.25 % of the various nitrogenous substances.

Table xi.—Sources of Nitrogen.

Phase	A2										B2									
Carbon source	Dextrose					Glycerin					Dextrose					Glycerin				
Days at 28°	3	6	9	13	19	3	6	9	13	19	3	6	9	13	19	3	6	9	13	19
Meat-extract	+	+	+	+	+	+	+	+	+	+	+	+	O	O	O	O	O	O	O	O
Peptone	+	+	+	–	O	O	O	+	+	+	S	S	O	O	O	S	O	O	–	+
Asparagin	+	+	+	+	+	+	+	+	+	S	+	+	+	S	O	S	+	+	+	S
Ammonium sulphate	+	+	+	+	+	+	+	+	+	+	S	+	+	+	+	+	+	+	+	+
Potassium nitrate	+	O	O	O	O	+	+	+	+	+	O	O	O	O	O	+	+	+	O	O
Potassium ferricyanide	O	+	+	O	O	O	+	+	+	S	O	O	O	O	O	O	O	O	O	O

Phase A1 is omitted from the table as all the tests were negative with the exception of peptone plus glycerine which became ropy on the 13th and was still ropy on the 24th day. On the 19th day a smear showed that the liquid contained 95 % of A2 and 5 % of A1.

On the 11th (B2) and 13th (A2) days the fluids were smeared on plates of nutrient agar and counts were made of the approximate proportions of the phases.

Table xi*a*.—Percentage Proportion of Phases.

Phase added	A2						B2					
Source of Carbon	Dextrose			Glycerin			Dextrose			Glycerin		
Phases determined	A1	A1 (tr.)	A2	A1	A1 (tr.)	A2	B1	B1 (tr.)	B2	B1	B1 (tr.)	B2
Meat-extract	—	100	—		90	10	dead			10	25	65
Peptone	30	70	—	25	40	25	—	—	100	75	—	25
Asparagin	—	25	75	—	65	35	15	—	85	—	—	100
Ammonium sulphate	80	10	10	—	90	10	—	100	—	70	30	
Potassium nitrate	dead			—	45	55	no growth			20	10	70
Pot. ferricyanide	10	80	10	—	—	100	nearly dead		100	—	—	—

It is difficult to see any relation between the ropiness as determined on Table xi*a*. with the proportion of the phases. Even when the transition phases of A1=A1(tr.) and of B1=B1(tr.) are included with the cohesive phases, there appears to be no reason for connecting ropiness with a particular phase of the organisms. One is, therefore, inclined to the idea that in most cases the ropy substance is formed first and the alteration of phase occurs subsequently (compare p. 64). The altered phase may in some cases digest the preformed ropy substance. The untabulated tests with phase A1 showed that the nature of the infecting phase largely determines the formation of ropiness.

Confirmatory tests were made with phase A1, a mixture of A1 with A2, and with A2 using levulose 2 % and sodium chloride 0.3 %. They bore out the results obtained with dextrose and citrate.

The work has shown that the phase A2 can produce ropiness from dextrose or glycerin in the presence of meat-extract, peptone, asparagin or ammonium sulphate, and from glycerin with nitrate but not from dextrose with nitrate. Doubtless this is due to the formation of an acid reaction in the medium containing sugar and the concomitant production of free nitric acid. Ferricyanide produced ropiness, but the quantity was scanty as compared with the other sources of nitrogen.

The phase B2 acted best with asparagin and ammonium sulphate. Meat-extract gave an evanescent ropiness with dextrose, but none at all with glycerin. The behaviour with nitrate was much the same as with A2 and probably for the same reason. Ferricyanide was an unsuitable source of nitrogen.

The Influence of Various Sugars, etc.

The activity of the bacteria in the presence of various sources of carbon was tested with a saline asparagin solution containing various sugars, etc. Ropiness was produced in the presence of saccharose, dextrose, levulose, galactose, glycerin and mannit. It was not produced from maltose, lactose, dextrin or gum-acacia.

In testing the most suitable amount of glycerin, a solution containing 1 % showed ropiness first, but in time the higher percentages made headway. On the eighth day the order of ropiness seemed to be 5 %, 1 %, 10 % and 2 % when the slimes were coagulated and weighed. With 1 %, 100 c.c. of media gave 86 milligrams, 2 % gave 64, 5 % gave 88, and 10 % gave 96 milligrams. The 2 % test was probably low, in which case all quantities gave much the same amount of ropy substance.

The ropiness seemed to become more abundant when grown in deep layers of fluid; in shallow layers there appears to be a greater growth of cells and less slime.

An experiment was made with carbohydrates when considering the subject of acidity. A solution containing sugar or glycerin 2 %, KH_2PO_4 0.2 %, $MgSO_4Aq.$ 0.1 %, $CaCl_2$ 0.02 % was made neutral to methyl-red and seeded with a mixture of A1 + A2 and with B2.

Table xii. Change of Reaction with Sugars and Glycerin.

Phase	A1 + A2					B2				
					End acidity to methyl-red					End acidity to methyl-red
Days at 28°	1	2	3	4	4	1	2	3	4	4
Dextrose	O	S	+	+	8°	O	O	O	O	9·5°
Levulose	O	+	+	+	9°	O	O	O	O	9°
Saccharose	S	+	+	+	6·5°	O	S	S	O	8°
Glycerin	S	+	+	+	9·5°	O	O	O	O	6·5°

The experiment showed a distinct advance of from +6° to +9° in the acidity with the sugars and a reduction of about the same number of degrees with glycerin during the four days' growth at 28°.

A more comprehensive test was made into the effect of various sources of carbon upon the production of ropiness. A medium containing 2 % of sugars, etc., 0.25 % meat extract and 0.5 % of crystalline sodium phosphate was prepared and portions were seeded with the phases.

Table xiii.—Sources of Carbon (1).

Phase	A1					A2					A2a					B2				
Days at 28°	1	2	4	6	9	1	2	4	6	9	1	2	4	6	9	1	2	4	6	9
Dextrose	O	S	O	O	O	O	+	+	+	O	O	O	S	O	O	O	O	O	O	O
Levulose	O	+	S	O	O	O	+	+	+	S	O	+	+	+	+	O	O	S	O	O
Saccharose	O	O	O	O	O	+	+	+	+	+	+	+	+	+	+	O	O	O	O	O
Glycerin	O	O	S	+	+	+	+	+	+	+	+	+	+	+	+	O	O	O	O	O
Lactose	O	O	O	O	O	O	S	S	S	S	O	S	+	S	O	O	O	O	O	O
Galactose	O	S	S	S	O	O	+	+	+	—	O	+	+	+	+	O	O	S	S	O
Mannit	O	O	O	O	O	+	+	+	+	+	+	+	+	+	+	O	O	+	+	+
Maltose	O	S	O	O	O	+	+	+	+	+	O	+	S	S	S	O	O	O	O	O
Dextrin	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
Gum acacia	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
Control	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

When the experiment was well under way, it was found that phase A2 and (A2a) had altered and contained more or less of A1, a fact that should be taken into account in considering the disappearance of the ropiness. It had also been discovered that the growth of phase A1 in glycerin caused some of the bacteria to assume the phase A2, possibly on account of the medium becoming alkaline in contrast to the acidification in the presence of sugars. The results of this experiment engender the belief that glycerin is the only substance of those tested which can alter A1 into A2 and that dextrose can rapidly alter A2 into A1. The role these substances play is presumably in the suppression or exaltation of the power of the bacteria to secrete a slime dissolving enzyme.

Meanwhile it had been determined that the saline constituents of the medium had an influence in the production of the ropiness, especially with phase B2. In a saline test, Table xvi., A1 gave a ropy medium in the presence of sodium chloride and with no other salt, while potassium citrate was most favourable with phase B2 and as good as several others with phase A2 (A2a). A medium was accordingly prepared containing 2 % of carbohydrate or other nutrient, 0.25 % meat extract and 0.2 % of common salt for phase A1 and of potassium citrate for the others. The bacteria had been picked from plates three days previously.

Table xiv.—Sources of Carbon (2).

Phase	A1 (NaCl)						A2 (Citrate)						B2 (Citrate)					
Days at 28°	1	2	4	5	7	11	1	2	4	5	7	11	1	2	4	5	7	11
1. Dextrose	O	O	O	O	O	O	+	+	+	+	+	-	+	+	S	S	O	O
2. Levulose	O	O	+	-	+	+	+	+	+	+	+	-	O	O	+	+	+	-
3. Saccharose	O	O	+	S	O	O	+	+		+	-	+	O	S	+	-	+	-
4. Glycerin	S	+	+	-	-	-	S	+	-	-	+	-	O	O	O	O	O	O*
5. Lactose	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
6. Galactose	-	+		O	O	O	+	-	-		+	-	O	-	-	+	+	-
7. Mannit	O	O		-	-	-	S	+	-	-	-	-	O	S	+	-	+	-
8. Maltose	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
9. Raffinose	O	O	O	O	O	O	O	S	O	O	O	O	O	O	O	O	O	O
10. No Sugar	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

* Confirmed by a separate test.

On the first day of the experiment, A1 (6), when plated, showed a pure culture of phase A1. On the eleventh day, B2 (1) contained no living bacteria in a large loop of the medium; tests A1 (2, 4 and 7) contained a mixture of the two phases, A1 and A2, as well as transition colonies.

A consideration of the last two experiments leads to the conclusion that in the presence of glycerin the phase A1 tends to become A2 irrespective of whether the salt is present as a phosphate or a chloride. With phosphate of soda and the various sugars the tendency is for it to remain unaltered. The case is different with sodium chloride, for levulose, and mannit (and also glycerin) change the phase to A2, the predominance of which gives a permanent ropiness within the limiting time of the experiment.

The phase A2 tends to change to A1 in the presence of dextrose and phosphate, but not so much with the other sugars, while in the presence of citrate it seems to remain unaltered.

Phase B2 (Table xiv.) is peculiar in giving no ropiness in the presence of glycerin and in the ropiness dissolving in the presence of dextrose. Dextrose appears to have caused the reversion to a phase B1 akin to A1 before assisting in the death of the organism.

The quantity of sugar has an influence in determining the ropiness of fluid media. For example a solution containing 0.25 % each of meat extract and sodium phosphate and 1, 2 and 5 % of dextrose showed the following with two cultures of A1. Phase A1(1) showed a colony with a granular centre. Phase A1(2) had a stippled centre. These had been picked from a plate thirteen days previous to the beginning of the experiment, and had been transferred daily in bouillon.

It is clear that 2 % and 5 % of dextrose are best for obtaining ropy solutions with races of A1 showing colonies with granular centres. The colonies with stippled centres have lost much of their slime-forming power. The stippling is

Table xv.—Increasing Amounts of Sugar.

Dextrose	1%						2%						5%					
Days at 28°	1	2	3	4	6	9	1	2	3	4	6	9	1	2	3	4	6	9
Phase A1(1)	S	S	O	O	O	O	+	+	+	+	O	O	+	+	+	+	+	O
Phase A1(2)	O	O	O	O	O	O	O	O	O	O	O	O	O	O	+	+	O	O
Acidity to methyl-red						+3°						−4°						+6°

caused by the presence of clusters of microscopic crystals of magnesium ammonium phosphate and these are not in evidence in the granular colonies. Large crystals, however, develop slowly in the agar, and are found in the old plates. It would appear that the development of ammonia runs *pari passu* with the formation of slime-digestive ferments, for it seems reasonable to consider that the ropy substance was digested as soon as it formed in the case of phase A1(2) with 1 % and 2 % of sugar. With 5 %, the increased sugar resulted in the slime-forming power temporarily overbalancing the slime-digesting power of the bacterium.

A synopsis of tables v., xiii., and xiv. gives a clearer view of the effects of the various sources of carbon than a detailed reference to the tables themselves. In this synopsis dextrin and gum-acacia have been omitted because under no circumstances did they ever assist in the formation of ropiness.

Table xiv*a*. – Synopsis of Sugar, etc., Experiments.

Phase	A1		A2				B2			
	Meat Extract with		Tannic Acid with		Meat Extract with		Tannic Acid with		Meat Extract with	
	phosphate	chloride	ammon. sulph.	asparagin	phosphate	citrate	ammon. sulph.	asparagin	phosphate	citrate
Galactose	S	+	−	+	+	+	+	+	S	−
Levulose	+	+	−	+	+	+	−	S	S	+
Saccharose	S	+	+	+	+	+	+	+	S	+
Mannit	O	+	+	+	+	+	S	+	+	+
Dextrose	S	O	+	+	+	+	+	+	O	+
Glycerin	−	+	+	+	+	+	−	−	O	O
Lactose	O	O		−	+	O	−		O	O
Maltose	S	O	+	O	+	O	O	O	O	O
Raffinose	−	O	O	O	−	S	O	O	•	O

Of all the sources of carbon, galactose seems best fitted to produce the ropy material. This is to be expected since the ropy substance is essentially a galactose anhydride and one would naturally think that the bacteria could form it most easily from this sugar. But the other sugars are not far behind in their capacity

for assisting in the production. Levulose and saccharose are goodseconds. There is a suggestion that sucrase or invertase is secreted by the bacteria as saccharose is the only biose that is utilised to any extent, but a search for this enzyme did not show its existence.

It is curious that the hexatomic alcohol, mannit, should be so good, but it seems to be peculiarly suitable for the production of many kinds of slime and is very frequently used in bacteriology for the nutrition of slime-forming bacteria.

Dextrose probably acts quite as well as the other substances, but it seems to be specially adapted to form a slime dissolving enzyme and on this account the rope-producing action of the sugar is not so clearly shown.

Glycerin is peculiar. It acts as a source of carbon for the phase A2 and alters A1 to this phase. It does not serve as a favourable nutrient for B2; indeed, it is not only unfavourable, but it also slowly changes B2 to the phase B1, and therefore acts in opposite directions with the two bacteria. Like mannit, it is a general nutrient for the production of slime from the majority of slime-forming bacteria, as will be seen from a perusal of my papers upon slime-forming bacteria.

Maltose, raffinose and lactose may be considered as being incapable of utilisation by the bacteria A and B. It is true that A2 can utilise them to some extent, but this phase is very active, and is able to make bouillon ropy, a fact which should be considered in connection with the production of ropiness in the presence of meat-extract.

It is probable that with suitable nitrogenous and saline nutrients and suitable conditions as regards acidity, the bacteria A and B are capable of forming ropy solutions from any source of carbon, and that the absence of the bacteria, rather than an unsuitable pabulum, should be the object aimed at in preventing ropiness in wattle bark infusions.

The Influence of Salts.

It is customary to add salts to bacteriological fluids for the purpose of supplying all those that may be necessary for the nutrition of the bacteria and of raising the osmotic pressure. The ordinary nutrient bouillon, agar and gelatine contains ½ % of common salt together with the salts that may be contained in the meat-extract used in the preparation of the media. With this amount of saline matter, the ordinary bacteria grow very well, but it does not follow that this amount is best for all bacteria. Water and soil bacteria, for example, exist upon much less, and it is a matter of common knowledge to the bacteriologist that the saline content of bacteriological fluids can be raised or lowered considerably without harming the bacteria to any great extent.

In the earlier experiments the saline matter had been usually added to the extent of 0.3 %, and generally consisted of potassium phosphate 0.2 %, magnesium sulphate, 0.1 %, and calcium chloride, 0.02 %. As these may or may not be good for assisting the bacteria in the production of the ropy substance, a number of tests were made to get some information upon the matter.

In an early experiment, a solution of glycerin, 2 %, and meat-extract, 0.25 %, was divided into portions, and each received 0.1 % of certain salts. They were seeded with A, a mixture of A1 and A2. That with calcium nitrate seemed to give the most slime on the second day. After twenty days' incubation the slimes were coagulated with alcohol and weighed. The milligrams of ash-free slime per 100 c.c. of liquid are given below:—

Calcium nitrate, aq.	260
Calcium chloride	175
Calcium lactate	152
Magnesium sulphate, aq.	132
Potassium monohydrogen phosphate	105
Sodium acetate	88
No salt	85
Sodium succinate	82
Sodium lactate	80
Potassium-sodium tartrate, aq.	57

The influence of the salts of the earths in promoting the formation of the ropy substance is clearly shown. Potassium, as represented by the phosphate, has more influence than the indifferent salts of sodium. The weights of slime obtained from the media containing the lactates of calcium and sodium show that the base and not the acid is the active component of the salt, but that the acid has some influence is indicated by the slime obtained in the presence of sodium-potassium tartrate.

These results were obtained in a medium containing glycerin, which in other tests had been found to maintain the original reaction or to bring about an alkaline condition of the medium. Dextrose and other sugars produced an acid condition and as wattle bark extracts are acid and as the carbohydrate in such extracts is probably of the nature of dextrose, possibly as a glucoside, it was considered advisable to test the activity of the bacteria in media containing this sugar with various salts. Accordingly a fluid containing dextrose 1 %, meat-extract 0.25 %, was prepared, and portions of it received 0.1 % of anhydrous salt. After sterilisation the sets were infected with bacteria which had been taken from pure colonies upon the previous day.

Table xvi.—Salts with Dextrose 1%.

Phase	A1			A2					A2a.					B2		
Days at 28°	1	3	5	1	3	5	7	13	1	3	5	7	13	1	3	13
Magnesium sulphate	S	O	O	S	+	+	S	O	+	O	O	O	O	O	O	O
Calcium lactate	O	O	O	O	S	S	S	S	–	S	O	O	O	O	O	O
Calcium chloride	O	O	O	S	–	+	+	O	+	+	S	O	O	O	O	O
Calcium nitrate	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
Sodium chloride	+	O	O	S	+	+	O	O	–	O	O	O	O	O	O	O
Sodium acetate	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
Sodium phosphate	O	O	O	+	+	+	+	S	+	+	+	O	O	O	S	O
Sodium lactate	O	O	O	S	O	O	O	O	+	O	O	O	O	O	O	O
Pot. sodium tartrate	O	O	O	S	+	–	+	+	+	+	+	S	O	+	O	O
Potassium citrate	O	O	O	S	+	+	+	+	+	+	+	+	O	+	S	O
Potassium nitrate	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
No salt	O	O	O	S	O	O	O	O	+	O	O	O	O	O	O	O

Certain of the tests were examined by plate culture from time to time, and it was found that A1 was pure on the 1st and 4th days. Phase B2 was pure on the 12th day. Phase A2a appeared to be a mixture of A1 and A2. Phase A2 seemed to be influenced in its persistence by the salt. In the sodium lactate test it was pure on the 12th day, in the citrate it contained a few of A1 on the 7th day and on the same day the sodium chloride test consisted chiefly of A1.

In the presence of sodium acetate and of calcium nitrate, not only was there no slime formed, but there was a complete absence of growth, a circumstance which led to the examination of the influence of the acetate upon the production of ropiness in bark extracts infected with rope-producing organisms.

The quantity of dextrose in the medium did not seem to affect the results to any degree, for the medium was strengthend with 3 % of dextrose and seeded with A1. Calcium chloride gave a slight and fugitive ropiness on the 1st day and citrate gave a fugitive ropiness on the 2nd day. All the other tests were negative.

As the activity of the saline constituents appeared to be of importance, especially in regard to the mutation of the organism, another test was made. In this the dextrose was used in 2 % strength with meat-extract 0.25 %, and the salts as before, viz., 0.1 % of the anhydrous salt. The infecting phases had been taken from typical colonies two days before the experiment was started.

Table xvii. Salts with Dextrose 2%.

Phase	A1					A2						A2a.						B2					
Days at 28°	1	2	3	7	10	1	2	3	7	10	17	1	2	3	7	10	17	1	2	3	7	10	17
1. Magnesium sulphate,	S	−	−	O	O	O	+	−	−	+	O	O	−	+	+	O	O	O	S	+	+	O	O	O
2. Calcium lactate,	O	O	O	O	O	O	+	+	+	+	O	O	+	+	+	−	S	S	O	+	+	+	S	S
3. Calcium chloride,	S	+	S	O	O	O	+	+	+	+	−	O	+	+	−	−	O	O	+	+	+	+	−	O
4. Calcium nitrate,	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
5. Calcium sulphate,	+	+	+	O	O	+	−	−	+	O	O	+	−	+	+	O	O	+	+	+	+	S	O	
6. Sodium chloride,	O	+	+	O	O	O	−	+	+	O	O	−	−	+	+	O	O	+	+	+	+	S	O	
7. Sodium phosphate,	O	+	+	+	O	+	+	+	+	+	+	+	+	+	−	O	O	+	+	+	+	−	O	
8. Sodium lactate,	O	O	+	O	O	O	+	+	O	O	O	S	−	+	O	O	O	+	+	+	+	O	O	
9. Sod. Pot. tartrate,	O	+	+	O	O	O	+	+	+	S	O	−	+	−	O	O	O	O	+	+	+	−	O	
10. Potassium citrate,	O	+	+	+	O	O	+	+	+	+	−	+	−	+	+	+	O	O	+	+	+	+	O	
11. Sodium succinate,	O	+	+	O	O	O	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	
12. No salt,	O	−	+	O	O	+	+	+	+	O	O	+	−	+	O	O	O	+	+	+	+	O	O	

On the seventh day some of the tests were plated with the following results:—

A1 (succinate), typical colonies of A1.

A2 (sod. lactate), colonies of A1 with stippled centres.

A2a (control), a mixture of colonies of A1 and A2.

B2 (magn. sulph.), colonies of B1, some with stippled centres.

On the tenth day other tests were plated.

A2 (phosphate), typical colonies of A1 as well as transition colonies of the same.

A2a (citrate), typical colonies of A1.

B2 (calc. sulph.), colonies of B1 with 2 % of B2.

B2 (phosphate), colonies of B1.

B2 (citrate), colonies of B1.

The disappearance of the ropiness in the test with magnesium sulphate first suggested the possibility of there being a phase of B2 secreting a digestive substance akin to A1, and the actual presence of this phase B1 upon the plates led to the examination of the stock culture. The latter was found to be pure B2 and the conclusion was reached that bacterium A was not peculiar in alone possessing phases or conditions with less physiological stability than races.

Part of experiment xvii. was repeated to confirm the changeability of phase B2, in media containing 1 % of dextrose, 0.25 % of meat-extract, and 0.1 % of anhydrous magnesium sulphate, sodium chloride or sodium lactate.

Table xviii.—Change of Phase.

Phase	A1					A2					A2a					B2				
Days at 28°	1	2	3	6	8	1	2	3	6	8	1	2	3	6	8	1	2	3	6	8
1. Magnesium sulphate	O	S	S	O	O	+	+	+	+	S	+	+	+	+	O	+	+	+	+	O
2. Sodium chloride	O	O	S	O	O	+	+	+	+	O	+	+	+	+	+	+	+	+	+	O
3. Sodium lactate	O	O	O	O	O	S	+	+	+	O	O	+	+	O	O	+	+	+	+	S

Plates were prepared on the eighth day.

A2 (magn. sulph.), A1, coarsely granular as well as transition forms.

A2 (sod. chloride), A1 with stippled centre.

B2 (magn. sulph.), B1, some with stippled centres.

B2 (sod. chloride), phase B1.

The experiment confirms the previous one, and shows the alteration of phases A2 and B2 into phases A1 and B1, in the presence of dextrose, some aid being possibly given by the salt.

Typical colonies of phase B2 were put into bottles containing dextrose with mixed salts and into glycerin with sodium phosphate, both with meat extract as a nitrogenous nutrient. No ropiness had occurred by the third day when plates were prepared. The glycerin contained phase B1 and B2 with transition colonies. The dextrose did not alter phase B2.

Typical colonies of phase B2 were seeded into fluid media containing 3 % of levulose or dextrose with 0.2 % of potassium citrate and 0.25 % of meat-extract. In four days the dextrose medium was ropy, while the levulose was not, and both contained the introduced phase in pure culture. By the seventh day the ropiness had disappeared in the dextrose flask, but plate cultivation showed that it contained the introduced cohesive phase B2 only. The levulose medium contained the cohesive and introduced phase B2, together with 25 % of the diffuse phase B1. The diffuse phase is akin to A1, but differs in being brownish or pale buff instead of yellowish or pale straw. Furthermore, there is the suggestion of

a wavy structure as if the flattened colony had an undulating surface. No further change had occurred by the sixteenth day.

The diffuse phase, B1, was grown in a glycerin phosphate medium to see in which direction an alteration would occur. On the 17th day, B2 was present, but it had disappeared by the 23rd. In another test the bacteria on the 6th day were all transition forms of B2, and on the 12th there were 75 % of B1, 15 % of B2, and 10 % of the transition form noted on the 6th.

The results seem to show that dextrose tends to maintain the phase B2 and glycerin the phase B1, but this is influenced by the nature of the salt.

Ordinary bouillon maintains the phase B2 and that is why the existence of a phase B1 was not suspected for a long time. It is different with A2 which is slowly changed to A1 in bouillon.

After finding that levulose favoured the production of the ropy substance more than dextrose, the experiment with the various salts was repeated upon two occasions using levulose as the carbohydrate. The phases A1, A2 and B2 were tested, and, for the sake of comparison, a further set of salts with dextrose was used for B2. The experiments duplicated one another, and the salient points of both are embodied in the table. Both sets of A1 (levulose) and B2 (dextrose) are omitted because they were almost entirely negative; the second set of A2 was the same as the first set.

After sterilisation, the dextrose medium in the first experiment had a reaction of $-1.5°$ to methyl-red and $+3°$ to phenolphthalein, and this was sufficiently alkaline to give precipitates in the tubes containing the salts of lime. In the course of the experiment, the reaction was tested on the seventh day in tests 4

Table xix.—Levulose with Various Salts.

Phase	A2			B2					B2 (Second Experiment)			
Sugar	Levulose			Levulose								
Days at 28°	1	3	12	3	5	7	10	12	3	5	9	14
1. Magnesium sulphate	S	+	+	O	O	S	+	+	+	+	+	S
2. Calcium lactate	S	+	+	O	O	O	O	O	O	O	+	+
3. Calcium chloride	O	+	+	O	O	O	O	O	S	S	+	+
4. Potassium sulphate	O	+	+	S	S	+	+	O	+	+	+	+
5. Sodium chloride	O	+	+	S	S	S	O	O	+	+	+	+
6. Calcium sulphate	S	+	+	+	S	S	S	O	+	+	+	+
7. Sodium phosphate	O	+	+	O	S	O	O	O	+	+	+	+
8. Sodium lactate	O	+	+	O	O	O	O	O	+	+	+	+
9. Sodium Potas. tartrate	O	+	+	S	S	S	O	O	S	S	+	+
10. Potassium citrate	O	+	+	O	S	S	S	O	+	+	+	+
11. Sodium succinate	O	+	+	O	S	S	S	S	+	+	+	S
12. No salt	O	+	+	O	O	+	+	O	+	+	+	+

and 5. Phase A2 with levulose showed +3° to methyl-red and +9.5° to phenolphthalein, while B2 with dextrose showed +11° and +14.5° respectively. Thus there was an approximate increase in the acidity during the seven days' incubation of +5° with levulose and +12° with dextrose. It is possible that the greater development of ropiness with levulose may be traced to the lesser production of acid favouring the stability of the cohesive phases of the bacteria.

Phase A1, in the first experiment, showed no ropiness in any of the tests until the 10th day, when that with succinate was ropy, and contained a mixture of phases A1 and A2a. In the second experiment, the tests were negative until the 5th day, when the phosphate gave a positive result. On the 13th day, the phosphate contained A2 with a few transition forms of A1. On the same day the potassium sulphate and sodium chloride tests contained the phase A1 with a few transition forms. On the 19th day, the ropiness had disappeared in the phosphate test, and the medium contained A2, 25 %, A1, 5 % and transition forms 70 %. In this case the phosphate apparently altered the phase to A2, which produced the ropy substance and, as the proportion of A2 decreased, the ropy substance dissolved. A1 has been omitted from the table.

Phase A2 produced ropiness with all the salts as well as in the control. It was apparently too active to require any assistance from the saline constituents. On the 12th day, the tests containing the salts of lime were gelatinous as well as ropy, and the media flowed like a soft jelly. In the second experiment, phase A2a was used, and all the tests were ropy on the 2nd day, and the ropiness persisted to the end of the experiment on the 19th day. Thus A2a duplicated A2.

Phase B2 with dextrose gave a slight ropiness on the first day with tartrate and succinate, but it had vanished by the 3rd day. Then all tests were negative until the 12th day, when the citrate test became ropy. In the second experiment no ropiness was obtained with any of the salts.

Phase B2 with levulose gave more favourable results, but there was a decided difference between the two experiments. That made on the later date gave a greater amount of ropiness which the control test seemed to indicate as being due to a more active condition of the infecting organism.

On the whole the saline tests, and especially those in the last two experiments, seem to indicate that given a suitable source of carbon and an active bacterium, the salts employed in the tests have little influence in producing ropiness. When the bacterium is not active, the salt may alter the phase, and thus assist in the production of a ropy liquid.

Acetates and Nitrates Check Ropiness.

The saline tests showed that nitrates and acetates prevented the development of ropiness in artificial media, and naturally this led to testing the influence of the acetate in bark infusions to see if the same prohibition occurred.

One part of bark was added to two parts of water and varying amounts of sodium acetate were added to the portions before seeding with B2. Ropiness developed in the control, but not in the portion containing 0.03 %, i.e., 3 parts per 10,000.

Another test was made with bark and water containing 0.02 % of acetate, portions being seeded with phases A1, A2 and B2. The controls became ropy.

and so did phase A2 with the acetate. The phases A1 and B2 with the acetate did not become ropy.

From these two tests, it appears that the limiting strength of the acetate for checking ropiness in bark infusions lies between 2 and 3 parts per 10,000.

Still one more test was made with bark and water containing 1, 2, 3 and 4 parts of acetate of sodium per 10,000, the liquids being seeded with phases A1, A2, and B2. Ropiness developed in the tests seeded with phases A2 and B2 containing one part per 10,000 but not in the stronger solutions. Phase A1 did not produce ropiness in the weakest solution.

The conclusion that we come to from a consideration of all the tests is that crystalline sodium acetate, when added to the water used for extracting wattle bark, in the proportion of three parts of salt to 10,000 of water or 3 pounds to 1,000 gallons will prevent the formation of ropiness in wattle bark extract.

The Nature of the Ropy Substance.

An attempt was made to obtain the slime in bulk by growing the bacterium A in fluid media containing dextrose or glycerin, but the quantities of slime were very small. This may possibly have been caused by autodigestion as noted in the various experiments with synthetic media, but of this I have no definite information to offer. More successful results were got by growing the organism on solid agar. Several drops of a broth culture of the organism was smeared on plates of a medium containing glycerin 5 %, meat-extract 1 %, potassium nitrate 0.1 % and sodium phosphate 0.2 %. The first growth obtained in a few days was yellow, loose, and was easily scraped off. The second growth that came up was translucent and elastic. It adhered with more or less tenacity to the agar, and some bits could not be removed. It was noted that the toughness increased with time, and the reason for this was explained later when it was learned that glycerin caused the phase A1 to alter progressively to A2, the more insoluble phase.

The collection of films was treated with alcohol and filtered; the coagulum was treated with water in which it simply swelled up; there was no solution. The swollen slime was heated in an autoclave at three atmospheres' pressure for half an hour when a solution and a sediment were obtained. The liquid was filtered with the aid of aluminium hydrate, and the filtrate was concentrated by evaporation. A portion sufficiently dilute to enable light to pass through was tested in the polariscope and found to give a reading of $+2.12°$ in a 200 mm. tube. Thus the gum was dextro-rotatory. The solution was further evaporated to a mucilaginous consistency and tested dropwise with various reagents.

Coagulation was effected with alcohol, basic lead acetate, ammoniacal lead acetate, ferric chloride and phosphotungstic acid, but the following had no action: lead acetate, baryta water, lime water, milk of lime, copper sulphate, the same followed by sodium hydrate, Fehling's solution, iodine, tannic acid, sodium hydrate or sulphuric acid. These are the general reactions with the autoclaved slimes, i.e., slimes which by the autoclave treatment have been separated into a soluble gummy matter and into coagulated proteid. The natural, uncoagulated ropy substance would behave quite differently. In one case where a slime was autoclaved for five and a half hours, coagulation was effected only with basic lead acetate and by phosphotungstic acid.

The soluble condition of the gummy matter does not appear to be stable, for when it was evaporated to dryness it became insoluble, and did not again form a solution with water.

The thickened mucilage which did not contain any reducing sugars, was boiled for ten hours with 5 % sulphuric acid under an aerial condenser, and during the hydrolysis it was noted that, like all the bacterial gums that I have examined, furfural was given off. The solution was neutralised with barium carbonate, filtered, treated with basic lead acetate, filtered, treated with sodium carbonate, again filtered, acidified with acetic acid, and evaporated. The solution was dextro-rotatory.

The osazone was prepared in the usual manner, and the bulk of the tar was removed by percolating the dried crystals with chloroform, then by a mixture of chloroform and alcohol, and finally with chloroform. The crystalline mass was dissolved in alcohol and allowed to stand. Successive crops of crystals deposited, and were removed, dried and tested for their melting points. These ranged from 202° to 193°. The intermediate crops were again crystallised, but in no case could crystals with a m.p. higher than 202° to 203° be obtained. Doubtless they were a mixture of glucosazone, m.p. 205°, and galactosazone, 193°, but the quantities were always too small to enable the pure glucosazone to be obtained. It is possible that the small quantity of glucose was present in the hydrolysed gums as an impurity. In testing the gum previous to hydrolysis for sugar, no positive indication was obtained, but it must be remembered that only a small portion was used and, while the impurity may not have been detectable in a small portion, it may show itself in the bulk after hydrolysis.

As an example of the relative amounts of crystals obtained, the following weights from a half portion of the hydrolysed gum are given.

1st crop—	12 milligrams,		201°
2nd „	—170	„	195°
3rd „	—138	„	193°
4th „	— 50	„	193°
5th „	— 27	„	193°
6th „	— 2	„	190°

Mother-liquor evaporated and treated with chloroform, which dissolved a brownish-yellow tarry matter.

residue .. .. 22 milligrams, 181°

The second bacterium, B2, was grown on plates of levulose asparagin tannin agar and yielded a number of tough skins which were easily pulled from the agar surfaces. It was not always possible to get the ropy material upon this medium for several later attempts failed. The slime of A2 is much more readily obtained. There was, however, sufficient slime to enable a determination of the hydrolytic products to be made. The rather thick emulsion, for the gum after solution by the autoclave treatment became partly coagulated upon evaporation, was unsuitable for testing the rotary power. The osazones were precisely similar to those furnished by A2, and yielded similar fractional crops of crystals melting at temperatures ranging from 202° to 193°, showing that the hydrolytic products of the slime of B2 were precisely similar in composition to those of A2.

The evidence goes to show that the ropy substance is essentially a dextrorotatory galactan.

A crop of films of the B2 slime of B2 was subsequently obtained upon an agar medium containing agar 2 %, saccharose 5 %, ammonium sulphate 1 %,

potassium citrate 0.3 % with 0.1 % of tannic acid added at the time of pouring the plates. After 17 days at 22°, the films were picked off, suspended in water overnight, and coagulated with alcohol. The water and alcohol treatment was repeated. The films suspended in water were heated in the autoclave for 15 minutes at three atmospheres pressure, but the treatment did not liquefy them. The water was acidified with two c.c. of normal sulphuric acid which produced an acidity of +5°, and the suspension was again autoclaved for an hour. The films had dissolved. The solution was carefully evaporated to smaller volume, and a portion was clarified with alumina cream and the rotation of the fluid observed. The ash-free solids had a specific rotation of $[\alpha]_D = +0.017°$. The solution gave a yellow precipitate with Fehling's solution, and it appeared that the treatment had partly hydrolysed the gum. It was treated with alcohol, and the unattacked gum was filtered off. The ash-free solids in the filtrate had a specific rotation of $[\alpha]_D = +0.002°$. The difference between these two rotations shows that the gum precipitable by alcohol is slightly dextro-rotatory.

The Acids formed by the Bacteria.

In the routine testing, the bacteria, A and B, were found to produce acid and gas from dextrose and saccharose when these sugars were present in broth. The nature of the acids was further examined. The bacteria were grown in a medium containing 5 % of dextrose, 1 % of meat-extract, and 0.5 % of sodium phosphate with the addition of chalk from time to time. The bacterium B2 used up the carbonate more quickly than A1 or A2, and naturally yielded a greater quantity of acids when the cultures were worked up at the end of a month's incubation.

The method followed in determining the nature of the acids, etc., was essentially that described in these Proceedings*

Ethyl alcohol was found in small amount in the cultures from both bacteria. It was proved by giving the iodoform test, by burning with a blue flame and by having a B.P. of 79°.

A small quantity of insoluble fatty acid was obtained from the culture of each bacterium. That from A melted at 37°, and from B at 32°. Both were probably mixtures, but the quantities were too small to separate. The softer acids of B were spread on a piece of filter paper and incubated at 28°, when the more fluid portion was absorbed, leaving a residue which melted at 40°, and became clear at 42.5°.

The volatile acids did not contain formic acid. The solutions were neutralised with baryta water, and after evaporation were dried at 140°. The A salts contained 52.36 % of barium, the B salts 53.8 %. As barium acetate contains 53.73 % of barium, it is clear that the volatile acids in both cases consisted entirely of acetic acid.

The non-volatile acids contained a small quantity of an acid giving a lime salt insoluble in 70 % alcohol. After acidification and extraction with ether, monoclinic prisms, melting at 182°, were obtained. Succinic acid under the same conditions melted at the same temperature, and thus it was proved that both bacteria form a small quantity of succinic acid.

The only other non-volatile acid was lactic. The zinc salt of lactic acid was prepared from two cultures of the A bacterium originally seeded with A1 and

Loc. cit., 1901, 606; 1903, 114.

A2. The first, A1, was separated as the lime salt from the non-volatile acids; the second was prepared directly from the total acids. A1 contained 18.12 % of water of crystallisation, and A2 contained 18.42 %. The latter showed a specific rotation of $[\alpha]_D = -3.35°$, and upon being acidified with hydrochloric acid in the proportion of 2 c.c. of strong acid to 20 c.c. of solution it showed no rotation. The acid was therefore inactive lactic acid with a laevo-rotatory zinc salt, and this was apparently the only form of acid present.

In preparing the zinc salt of the B2 acid, three crops of crystals were obtained. The first weighed 2.02 grams, and contained 13.26 % of water of crystallisation. Zinc paralactate contains 12.9 %, equivalent to two molecules. The zinc salt when dissolved in water had a specific rotation of $[\alpha]_D = -5.18°$, and with the addition of 2 c.c. of strong hydrochloric acid to 20 c.c., the rotation became $[\alpha]_D = +2.74°$. The first crop of crystals therefore consisted of paralactate. This acid is said to be contained in meat-extract, but in this case it was the result of the bacterial activity, because it was not found in the cultures from Bacterium A which was grown in media prepared from the same formula.

The second crop of crystals weighed 0.8 grams, and contained 15.18 % of water of crystallisation, showing it to be a mixture of two forms of acid. The third crop weighed 0.36 grams, and contained 18.75 % of water. The zinc salt of ordinary ethylidene or fermentation lactic acid contains 18.18 %, equivalent to three molecules of water, and this was undoubtedly the form of acid in the third crop of crystals.

The calcium salt was prepared from a portion of the non-volatile acids. It contained 26.56 % of water, equivalent to 4½ molecules (26.2 %), and was either a mixture of the calcium salts of the two forms of acid, or it was the more insoluble paralactate, as was indicated by the comparative quantity obtained (2.9 grams). The calcium salt of the ordinary acid would probably have been in the mother liquor from the crystals.

The acids formed by the two bacteria, A and B, from dextrose in the presence of chalk have been shown to consist chiefly of lactic and acetic acids with small quantities of succinic acid and mixed insoluble fatty acids. Ethyl alcohol was also formed in small amount, and it may be that this was the source of the acetic acid. There was a difference in the nature of the lactic acids. Both bacteria formed the ordinary fermentation lactic acid, but B2, in addition, produced the dextro-rotatory paralactic acid.

A Glucoside may be formed.—When the A2 culture was acidified with sulphuric acid and extracted with ether, a quantity of films was carried up by the ether and conveyed to the distillation flask. At the end of the extraction, the ether was shaken up with water and the supernatant ether containing the acids was used for their identification. The yellowish watery liquid was evaporated, and yielded a syrup which was assumed to be glucose carried over with the films. Upon tasting it, however, it was found to be intensely bitter. The syrup was diluted with water, acidified with acid and shaken up with chloroform. The chloroform was evaporated off, and a yellow bitter syrup obtained. The acid solution was treated with ammonia in excess and again extracted with chloroform. Upon evaporating the chloroform, a small quantity of a colourless bitter syrup remained. The presence of a glucoside is therefore indicated, and should this prove to be correct, the further examination will be dealt with in a future paper.

Cultural Characters.

BACTERIUM A, with phases A1 and A2, A2a.

Morphology.—A Gram-negative, motile, short rod with rounded ends. It appears generally as a rod $0.5 \times 1\mu$, but varies from an apparent coccus to rods up to 2μ in length. Spores were never observed. The flagella are long and vary in number. They are frequently single, and polar, but more often they are peritrichous. Up to five have been observed.

Nutrient agar stroke.—A raised, glistening, canary-coloured growth of loose consisteney. The cohesive phase A2 grows as a dry rough expansion.

Nutrient agar colonies.—After a day's incubation at 28° there is little distinction between the phases beyond the tints under the microscope. A1 is yellowish, A2 is grey, and all phases are either homogeneous or have a finely granular centre. Differences are readily seen on the second day, when A1 is circular, slightly raised and yellowish, while A2 and A2a are milky white and dome-shaped. A2a maintains the dome shape, but A2 has developed or will develop a more or less flattened and corrugated base, so that the whole colony has a nipple-shape. In consistency A1 is quite loose, A2 and A2a are ropy or tough, and adhere firmly to the agar from which the colony has to be dug away. A2a is more ropy than A2. Microscopically, A1 is canary-coloured, A2 and A2a are smoke-coloured or grey. A1 has a granular centre with homogeneous outer portion. A ring of egg-shaped granules is frequently seen around the centre among the smaller granules which become finer and ultimately vanish in the homogeneous portion. The granulation may be replaced by a stippling due to the presence of small clusters of crystals of triple phosphate. In old plates, four or five days, the agar becomes studded with comparatively large aggregates of the same crystals. A2a is round, has a dark centre and a cog-wheel structure at the margin. In some cases the centre is lighter, and a rosette structure can be made out. A marginal ring shows protrusions which alternate with the points of the rosette giving rise to the cog-wheel appearance.

A2 is not rounded or circular like A2a, but is more or less roughly dentate. There are usually from five to seven lobes, more or less roughly pointed, and the rough points consist of frog-spawn-like masses of granules. The internal structure is not visible, but there is an occasional suggestion of a rosette or radial structure.

Divergences from these phases have been noted as transition forms. The main difference between A1 and A2 is in the colour, the difference between a canary colour and a smoke tint. The yellow transition colonies range from the more or less pitted forms of the stippled or granular colonies of A1 to those in which the whole colony is granular with the granules radiating to the edge and becoming more and more coarsely granular as the margin is approached. The smoke-coloured transition colonies show a fibrous structure, the coarse fibres stretching from a dark centre to near the margin. Some colonies have been seen with this fibrous structure at one side and the A2 structure at the other.

The difference in microscopical structure is closely associated with the flat, dome or nipple-shaped macroscopical structure of the colony.

When the bacteria have been quiescent for some time, as, for example, when they have been existing upon agar or in broth for a month or two without transfer, these differences may not be noted. Raised, flat-topped colonies may form, and these do not show any characteristic markings.

Nutrient-gelatin stab.—In three days, A1 showed a filiform canal and sunken nail-head. In five days there was a liquefied saccate area at the top of the canal.

A2 and A2a showed a filiform canal with an upper portion waved and bearing a flat nail-head. In five days the nail-head had become a napiform softened area.

Nutrient gelatin colonies.—A1 gave colonies showing an irregular, granular, ivy-leaf-like structure in a shallow depression of softened gelatin. By the fifth day the gelatin had liquefied and the growth had broken up into irregular scattered granules. A2 and A2a liquefied the medium slowly, and the colonies remained as moruloid or frog-spawn-like masses of irregular granules.

Glucose gelatin colonies.—The phases were all much the same, and this applies to all media with sugar. A1 gave pale yellow colonies with raised centres and raised circular margins (button-shape); they were about 7 mm. diameter in four days. A2 grew as irregular moruloid masses, 3-5 mm. in diameter. Both phases softened the gelatine.

Dextrose agar.—A1 grew as a smooth raised colony of ropy consisteney; A2, dome-shaped, with or without a rugose margin, and the consisteney was rubber-like rather than ropy.

Bouillon.—A pronounced surface film and slightly turbid medium with a faint deposit excepting when a film has fallen down. A2 causes the upper layers of medium to be ropy. Nitrates are reduced to nitrites, indol is formed and ammonia is produced.

Potato.—A scanty, glistening, pale buff growth.

Starch.—Faint saccharification occurred.

Litmus-milk.—The medium was unaltered.

Litmus broth with sugars, etc.—Saccharose and dextrose gave acid and gas. Mannit showed a bleaching only, lactose was unaltered.

Classification number.—221.1313523.

Bacterium B2.

Morphology.—As A2, but a little stouter rod, 0.6 μ.

Nutrient Agar Stroke.—As A2.

Nutrient Agar Colonies.—A corrugated, dome-shaped colony smaller than A2, in appearance like a minute white raspberry. Microscopically, the colonies on thickly sown plates show a granular central area bounded by an irregular, dark, ivy-leaf-shaped band outside which and half way to the edge there is a dark circular ring; otherwise the colony structure is coarsely granular. The freely-growing colonies have often rosette or spoke-like markings extending from the centre to the repand edge, but the typical structure is mesenteric.

Nutrient gelatin stab.—As A2, but the liquefaction is very slow.

Nutrient gelatin colonies.—As A2.

Glucose gelatin colonies.—As A2, but they do not liquefy the medium.

Dextrose Agar.—As A2.

Bouillon.—As A1, but the film is flakey.

Potato.—A glistening white growth.

Starch.—As A.

Litmus milk.—As A.

Litmus broth with sugars, etc.—As A.

Classification number.—As A.

The two bacteria have some resemblance to *Bac. Atherstonei*, the variable galactan bacterium described by me as having been obtained from the tissues of *Strychnos Atherstonei*.* That organism exhibited two phases. The colonies in glucose-gelatin grew as brittle transparent masses, apparently containing a brittle

*These Proceedings, 1904, 442.

transparent gum, and as loose, yellow, slimey growths. The cohesive phase was rapidly changed to the diffuse phase by growing in glucose-gelatin at 30°. The gum was a galactan, but was hydrolysed with difficulty, while the reactions of the mucilage were different from those noted with the wattle-bark bacteria.

Conclusions.

The investigation was undertaken with the idea of endeavouring to elucidate one of the problems that is occasionally met by the tanner. It is possible that every case of ropiness may not be bacterial, but it may be granted that in the great majority of cases it is a bacteriological phenomenon, and any information regarding it should be of value.

To attack the problem from the side of the tannery would be a matter of much difficulty, for one cannot always get cases of ropiness at suitable times, and, when ropiness does occur, circumstances may not be such as to facilitate the investigation. That it is not an easy problem is shown by the fact that up to the present it has not been investigated, and, doubtless, this may be traced to the multiplicity of organisms swarming in the tan-liquors and the habit which slime bacteria have of growing in clumps, while most of the other bacteria diffuse themselves. It appeared to be an easier way to attack the problem from another aspect, that of the ropiness that occurs in wattle bark infusions, and there is every reason to believe that the results obtained with the infusions will be largely applicable to tanning liquors.

In the bark of wattle trees, many bacteria may be capable of producing ropy infusions, but so far only two have been found. They are closely allied to one another and differ, not so much in their bacterioscopic characters as in their physiological properties, that is, in their power of forming the ropy substance under different conditions, especially as regards nutrition. The bacteria have been provisionally named A and B. Like several gum-forming bacteria which have been described by the writer,* each bacterium can show two phases, one forming a comparatively soluble slime, the other giving a viscous slime.

The possession of two phases is not unique and possibly a double phase may be expected to occur with many slime-forming bacteria. The alteration of phase is possibly associated with the presence or comparative absence of a gum-digesting enzyme. The soluble phase certainly possesses a larger amount of a gelatine-dissolving enzyme, and one can, at will, by altering the incubation temperature, obtain a mobile or a viscous fluid.

There appears to be something in bark infusions that induces the soluble phase to become the insoluble phase and gives rise to ropiness.

The tannins of bark infusions have a prohibiting action upon the formation of ropiness, on account of their property of coagulating the slime, and for this to occur a certain concentration is necessary. For Bacterium A this is equivalent to a specific gravity of 1.024, and for Bacterium B, 1.048. Pure tannic acid is more active, for the prohibiting amount is much under the quantities of tannin represented by the gravities of the infusions. In synthetic media, 2 % of tannic acid prevents the formation of ropiness by coagulating the slime, as it is formed, upon the bodies of the bacteria.

In view of this differential action of tannin as compared with tannic acid,

*The bacteria responsible for the production of the soluble and insoluble wattle gums were named *B. acaciae* and *B. metarabinum*, and it was shown that the one form could be altered to the other. *B. Athersloniei* (these Proceedings, 1904, 442) exists as two phases, one forming a soluble slime, the other producing an insoluble gelatinous galactan.

the liability of weak tanning end liquors to become ropy will depend upon the tannic acid, for the tannins will have become hydrolysed by bacterial action to glucose and tannic acid. Thus a fresh liquor with a sp. g. of, say, 1.024, containing about 5% of tannin will be quite different in its action to an old liquor of the same gravity containing perhaps 5 % of tannic acid. Again, it has been shown that certain salts, notably acetates, prohibit the growth of the rope-forming bacteria. Acetic acid is a very common by-product of bacterial activity, and may follow up the alcoholic fermentation should yeasts become active in the liquors. If acetates are present in the spent liquors they will have a decided influence in preventing the development of ropiness. The matter is therefore complicated; so much will depend upon the composition of the liquor.

Once the ropiness is formed in infusions still in contact with the bark, it does not disappear even although the concentration of tannin becomes greater than that necessary to coagulate the slime. Under similar conditions in synthetic media, or in infusions out of contact with the bark, the ropiness disappears either through coagulation or digestion.

The acidity of the infusion doubtless plays a part in promoting ropiness. This was the case with synthetic media which with some phases of the organisms gave most ropiness when the acidity varied from +8 to +12°, or when it contained from 0.75 % to 1 % of tannic acid. In opposition to this, the bacteria when grown in the presence of chalk and, therefore, in a neutral medium, produced ropiness at 28°, and not at 37°.

But the main condition is the presence of a sugar and of the many that were tested, galactose was the most efficient in promoting the formation of mucus. Levulose, saccharose, dextrose and the non-sugars, mannit and glycerin were nearly as good, while maltose, lactose and raffinose were incapable of assisting the slime-forming function.

The nature of the salts did not appear to have much influence when sufficient sugar was present. But with a deficiency of sugar (1 %) or with a feeble bacterium, the salt may play a part. Acetates and nitrates prevented the growth of the active bacteria, and they give us a means of preventing the development of ropiness in bark infusions and presumably in tanning liquors. Three pounds of acetate of soda to 1,000 gallons of the water used in making the extract will prevent the development of ropiness.

So far as the nitrogenous food is concerned, it did not seem to matter much whether meat-extract, peptone, asparagin or ammonium sulphate was used. Nitrates in alkaline solution will also serve, but in the presence of acid or what comes to the same thing, in the presence of sugar, they prohibit growth.

The ropy substance itself is a galactan, and by the hydrolytic action of sulphuric acid is converted to galactose. The insoluble slime swells up enormously with water, and in common with most insoluble gums, can be liquefied by heating under pressure in contact with a small quantity of sulphuric acid (+3°).

Certain by-products are formed by the bacteria when growing in solutions of dextrose and saccharose in the presence of chalk. These consist of ethyl alcohol, succinic acid, a mixture of fatty acids, all in small amounts, and acetic and fermentation lactic acids. The lactic acid preponderates. In addition to these, which are formed by both bacteria, Bacterium B produces paralactic acid.

Other differences between the bacteria A and B are that B does not seem to be able to utilise glycerin, and its insoluble phase, as compared with A, is very stable.

SUMMARY.

Two closely allied bacteria were isolated from ropy infusions of wattle bark. They caused the mucinous fermentation of bark infusions and of synthetic media containing sugar.

Fresh infusions, of Sp.G., 1.024 and less, were made ropy by A, and of 1.048 and less, by B.

The bacteria exist in two phases which can be altered at will. One produces a soluble slime, the other an insoluble mucus. The ropiness is produced chiefly by the insoluble phases.

The utilisable sugars are galactose, levulose, saccharose and dextrose, the non-sugars are mannit and glycerin.

The sources of nitrogen include meat-extract, peptone, asparagin, ammonium sulphate, and potassium nitrate in alkaline solution.

The saline constituents have little influence in presence of sufficient sugar.

A slight acidity favours the production of ropiness, the optimum ranging from + 8° to + 12°. The optimum amount of tannic acid runs from 0.75 % to 1 %. The limiting amounts are +30° and 2 % of tannic acid.

The mucus is a galactan, and is hydrolysed to galactose.

The by-products from sugar are chiefly inactive lactic and acetic acids. Ethyl alcohol, succinic acid and a mixture of non-volatile fatty acids are produced in small amounts. In addition to these, bacterium B produces paralactic acid.

I have to thank Mr. F. A. Coombs for information regarding the use of wattle-bark and for obtaining the opinions of some tanners upon the occurrence of ropiness. I am also indebted to Mr. W. W. L'Estrange for much valuable assistance given during the course of the investigation.

APPENDIX:—*Opinions upon Ropiness.*

Mr. F. A. Coombs, Lecturer upon Tanning in the Sydney Technical College, circularised a number of master tanners asking their experience regarding the occurrence of ropiness in wattle bark liquors, and the replies are thus summarised

Ropiness does occur in wattle bark liquors, but as to its frequency in barks from particular places or from young or old trees no information could be obtained.

It occurs in liquors prepared from immature or freshly-stripped bark.

It is met with most frequently during the Summer months, January, February, and March, but may also occur in the Spring.

Ropiness may develop in weak or strong liquors.

If the liquors stand for a fairly long time without handling they may become ropy.

It may not be attributed to the constant use of spent colouring liquors, but this presumes that they have been treated in some way. One tanner was definite in stating that the trouble starts with the use of weak colouring liquors that ought to be run away, and added that possibly some tanners, when strengthening the spent liquors, let the bark ferment.

Ropiness occurs in liquors other than wattle-bark liquors.

The weak or spent liquors, when not run away, are either steamed, boiled, or treated with disinfectant, and in these ways the development of ropiness in the liquors is prevented.

EXPLANATION OF PLATE IX.

Colonies growing on the surface of Nutrient Agar.

1.—Colony of A1.

2.—Colonies of A1 and A2a growing side by side. The almost homogeneous character of A1 and the cog-wheel structure of A2a are brought out.

3.—Colony of A2. This was a specially translucent colony. They are generally opaque, except at the margin.

4.—Colonies of B2. Thickly sown colonies, showing the ivy-leaf structure.

5.—Colony of B2. Mature colony, showing the mesenteric structure.

6.—Ropy Bark infusion, flowing siphon-wise.

(Photographed by Mr. W. W. L'Estrange.)